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Use of verapamil and verapamil derivatives for the preparation of pharmaceuticals with β-glucuronidase-inhibiting action in human tissue

The subject of the present invention is the use of verapamil or verapamil derivatives in pharmaceuticals for the inhibition of the enzyme beta-glucuronidase in human tissue with the object directly to achieve therapeutic effects or to improve its therapeutic breadth by combined use together with glucuronidated or glucuronidatable active materials.

The conjugation of endogenic or exogenic substances with glucuronic acid is an important metabolic reaction in humans and animals. Glucuronic acid can be conjugated with the most varied substances, e.g., pharmaceutically active materials and their metabolites. The conjugation reaction takes place by transfer of activated glucuronic acid (UDP-glucuronic acid) to the substrate by means of the enzyme glucuronyl transferase. In general, the organism uses the conjugation reaction for detoxication since glucuronic acid conjugates are usually less toxic and, on the basis of their good water solubility, are easily excreted via the kidneys or the gall secretions via the intestines. A conjugation can also take place in non-enzymatic ways by chemical synthesis.

The glucuronic acid conjugates can, however, also b cleaved by catalytic action of glucuronidases into glucuronic acid and into the starting product. The cleavage of glucuronides frequently takes place after excretion thereof via the bile in deeper lying small intestine sections or in the large intestine. The thereby resulting starting substances can again be resorbed and this become renewed active in the organism. This process, designated as enterohepatic circulation, can prolong the desired action of substances but can also increase the toxic actions of poisonous substances.

By medicamentous regulation of the beta-glucuronidase activity in the various tissues, new therapy concepts are opened up.

Use of glucuronidase inhibitors in cancer therapy.

A peculiarity of cancer tissues is their high concentration of beta-glucuronidases or an extremely high glucuronidase activity. Closely associated with the increased glucuronidase activity is the tendency to form certain tumour metastases. By general administration of a beta-glucuronidase inhibitor, in the case of tumours which, on the basis of the increased beta-glucuronidase activity, tend to the progression and metastasis formation, the tumour spreading out is reduced via the inhibition of the tumour glucuronidase. Saccharo-1,4-lactone, 2-acetamidoglycal and heparin derivatives were tested for this purpose (Bernacki R.J., Cancer Metastasis Rev., (1985) 4: 81 - 101; Nakajima M., Journal of Cellular Biochemistry (1988) 36: 157 - 167; Niwa T., Journal of Biochemistry (1972) 72: 207 - 211). In most recent times, selective glucuronidase inhibitors have been synthesised (Bosslet K., EP 0822192).

Besides the general use for the therapy, glucuronidase inhibitors can also be used supportingly in the chemotherapy of cancer patients for the increasing of the desired effect in the case of simultaneous reduction of the undesired actions.

The chemotherapy causes an extraordinary physical and psychic stressing of the cancer patient. Glucuronidase inhibitors can ameliorate negative actions of the chemotherapy and simultaneously increase the effectiveness of the therapy. For this purpose, the following starting points present themselves.

Chemotherapeutics are, inter alia, also excreted via their glucuronides via small intestines. Due to the actions of the there-present glucuronidases, there takes place a cleavage of these glucuronides and liberation

of the active cell-toxic substances which damage the intestinal tissue present in continuous cell division and regeneration. For the patient, there result therefrom nausea, vomiting and diarrhoea, combined with a fluid and weight loss.

Beta-glucuronidase inhibitors can protect the intestines against toxic products from cytostatic glucuronides. Thus, e.g., the intestinal toxicity of the anti-tumour agent irinotectan hydrochloride can be minimised by preventative administration of the beta-glucuronidase inhibitor baicalin. The patients are thus protected against a massive diarrhoea and the fluid losses involved therewith (Takasuna K. Jpn. Cancer Res. (1995) 86: 978 - 84; Kamataki T. U.S. Pat. 5,447,719).

Considerations exist of using the cleavage of glucuronides in certain tissues in order to liberate the active substances from inactive precursors of active medicaments (prodrugs). Due to the preferred liberation in the diseased target tissues, via the increased substance concentration, there can be achieved a more or less local action in the case of low systemic action (Sperker B., Clin. Pharmakinet. (1997) 33: 18 - 31). This therapy possibility would be of interest above all in the case of the use of side effect-rich substances in tumour therapy because the desired cytotoxic properties of chemotherapeutics can be concentrated on the tumour tissues. The tumour progression and the metastasis formation is frequently bound up with an increased glucuronidase activity. In necrotic tumour regions, an increased glucuronidase activity is present in the extracellular space whereas in the healthy tissue the glucuronidase activity is substantially intracellular localised. A pH value in the tumour displaced towards acid can again increase the activity of the beta-glucuronidase. These physiological conditions offer starting points for the application of glucuronic acid conjugates with chemotherapeutics to tumour patients for the local

liberation of the active substrate after cleavage by the locally increased glucuronidase activity (Sperrker B., Clin. Pharmacokinet. (1997) 33: 18 - 31). The local action could be strengthened by simultaneous administration of a glucuronide prodrug and of a tumour-specific antibody which is covalently bound with beta-glucuronidase (antibody-directed prodrug therapy = ADEPT) (Sperker B., Clin. Pharmacokinet. (1997) 33: 18 - 31).

The increased tumour selectivity of glucuronide prodrugs leads to correspondingly higher active material levels in the tumour and sumultaneously to lower active material concentrations in healthy tissue regions, i.e. the effectivenesses and compatabilities of the chemotherapeutics are increased.

Known examples are doxorubicin glucuronide prodrugs which, in comparison with the free doxorubicin, make possible in tumour tissues an about 10 times higher doxorubicin level but, at the same time, protects healthy tissue with a lower concentration so that e.g. the typical cardiotoxic property of doxorubicin only plays a subsidiary role (Bosslet K., Cell Biophys. (1994) 24-25; 51-63; Bosslet K., Cancer Res. (1994) 54: 2151-9; Bosslet K., Cancer Res. (1998): 1195 - 201; Murdter, T.E., Cancer Res. (1997) 57: 2440-5).

None of these investigations has hitherto lead to therapeutically usable results, i.e. utilisable medicaments.

Description of the invention

The invention has set itself the task of finding glucuronidase inhibitors which are otherwise pharmacologically not or only little effective, i.e. display few side reactions, in order to use these as medicaments in the above-described uses alone or in combination with other medicaments for the increasing of the therapeutic breadth.

This task is solved by the features of the main claim and promoted by the features of the subsidiary claim.

It is known that verapamil inhibits the activity of bacterial beta-glucuronidase (E. coli) to a considerable extent (B. Sperker et al., Eur. J. Clin. Pharm. (1999), Vol. 55, A, 16) but does not inhibit the glucuronidase in the intestinal tissue of rats (mammals) in contradistinction to known glucuronidase inhibitors, such as D-saccharic acid 1,4-lactose, which, in the case of the rat enzyme, inhibits 30 times more strongly than the enzyme from E. coli.

Surprisingly, it has now been found that verapamil exerts a strong inhibiting action on the β-glucuronidase occurring in the human tissues. The inhibition takes place in the case of an administration of 1 - 10 mg per kg body weight and day to an equal extent by the racemic mixture and the pure enantiomers. It is known that the diverse actions of verapamil, known as calcium antagonist, on the heart and vascular system essentially come from the S-enantiomer (Mickisch G.H., J. Cancer Res. Clin. Oncol. (1995) 121 (Suppl. 3): R11 - R16). Thus, in the case of the scarcely cardioactively effective R-enantiomer of verapamil or verapamil derivatives, the desired inhibiting effects on the beta-glucuronidase activity are achieved without the pharmacological actions known for verapamil occurring as undesired side effect.

In particular, the adjuvant oral administration of retarded medicaments of verapamil or its derivatives is intended for uses which, over comparatively long periods of time, are to protect the intestines against the toxic cleavage products for less toxic β-glucuronides. In the case of adjuvant administration in cancer therapy, the thereby also occurring systemic distribution of the inhibitors of the verapamil type is no disadvantage. It is known that verapamil favourably influences the treatment

of chemotherapy-resistant cancer cells (Volm M., Anticancer Res. 18 (C4): 2905 - 17; Wainer I.W., Ann. Oncol. (1993), 4 (Suppl. 2): 7 - 13). Various mechanisms of the manner of working are thereby discussed, whereby verapamil suppresses the active passing out of the chemotherapeutic from the cancer cells (Simpson W.G., Cell Calcium (1985) 6: 449 - 67) or perhaps prevents the expression of multidrug resistance genes (Ling V., Cancer Chemother. Pharmacol. (1997) 40 (Suppl.): S3 - S8; Mickisch G.H., J. Cancer Res. Clin. Oncol (1995) 121 (Suppl. 3): R11 - R16). A participation of β-glucuronidases is not given the case of these mechanisms.

Glucuronidase inhibitors of the verapamil type can also be used supportingly in chemotherapy together with novel glucuronide prodrug chemotherapeutics. The therapy supporting with glucuronidase inhibitors of the verapamil type comprises the protection of the healthy tissue against the actions of these chemotherapeutics, especially against the actions of higher local concentrations at injection points or other places of introduction.

The verapamil administration and dosing takes place in such a way that locally at the infusion entrance the healthy tissue is protected, i.e. the glucuronidases are there inhbited but, after the systemic mixing up, no deactivation of the tumour glucuronidases takes place in the tumour tissue.

Physiologically less stable glucuronide prodrugs can pharmaceutically be so stabilised by addition of the glucuronidase inhibitor verapamil that only after the systemic mixing up in the organism does the cleavage preferably take place in the target tissue.

In the case of administration of biologically-inactive glucuronide prodrugs, together with beta-glucuronidase inhibitor, the cleavage into the effective substrate is delayed so that, in the case of prodrugs with long elimination half value time, the systemic availability

is prolonged. Correspondingly, the dose can be reduced and the dosaging interval lengthened.

In the case of the tumour-specific prodrug therapy, by additional administration of a cell membrane-permeable beta-glucuronidase inhibitor, such as verapamil, the therapeutic breadth is thereby increased that the substantially intracellularly present beta-glucuronidase is inhibited in healthy tissue and a pharmacological action is thereby hindered. In the tumour tissue, due to the physiological or due to the glucuronidase concentration increased by ADEPT therapy, the effective substrate is, as previously, formed in the case of suitable choice of dose.

The inhibiting action on the beta-glucuronidase activity claimed in the invention is verified in the results set out in the following.

Investigations of the lowering of human β-glucuronidase activity by verapamil, its metabolites and gallopamil.

The calcium antagonist verapamil (not only racemate but also both enantiomers), its metabolites and the derivative gallopamil are in the position to lower the activity of the human β-glucuronidase.

A direct inhibition of the β-glucuronidase activity could be shown in experiments with human liver homogenates. For this purpose, homogenates of various liver samples were incubated with 2.5 mM 4-methyl-belliferyl-β-D-glucuronide (MUG) and analysed by means of HPLC. The concentrations of the liberated 4-methylumbelliferone is a measure of the activity of the β-glucuronidase. In the case of homogenates which, in addition to MUG, also received 100 µM verapamil (racemate), the activity was reduced significantly by about 25%, in comparison with the control samples (Fig. 1).

Parallel bring about verapamil, the metabolite norverapamil, D702, D 703 and gallopamil in the human hepatoma cell line HepG2 after 48 h incubation a reduction of the β-glucuronidase activity to 50 - 65% which is to

be attributed to a reduced expression of the enzyme. This reduction of the activity is concentration dependent (Fig. 2).

The reduction of the β-glucuronidase activity could be observed equally strongly with verapamil racemate and with R- and S-verapamil. The metabolites norverapamil, D 702 and D 703 show a comparable influence on the activity of the β-glucuronidase in HepG2 cells. The incubation with D 617, a further metabolite, only brings about a lowering of the activity by 12% which, however, is not statistically significant. Gallopamil brings about an effect comparable to verapamil (Fig. 3).

Example 1

Inhibition of the activity of human liver β-glucuronidase by verapamil (Fig. 1).

Human liver homogenates were incubated with the enzyme substrate 4-methylbelliferyl-β-D-glucuronide (1 h, 37°C). 100 µM verapamil or DMSO (control) were added to the reaction mixture. The liberation of 4-methylumbelliferone was measured by means of HPLC analysis (* significant difference to the control; $p < 0.001$; n = 3 independent experiments).

Example 2

Concentration dependency of the verapamil action in the human hepatoma cell line HepG2 (Fig. 2).

HepG2 cells were incubated for 48 h at 37°C with the concentrations of verapamil given in Fig. 2. After lysis of the cells, in each case 2.25 µg of cellular protein were incubated (2 h, 37°C) with the glucuronidase substrate 4-methylumbelliferyl-β-D-glucuronide and the concentration of the liberated 4-methylumebelliferone measured by mean of HPLC (* significant difference to the control, $p < 0.05$).

Example 3

Lowering of the β-glucuronidase activity in HepG2 cells by incubation with verapamil, verapamil metabolites and gallopamil (Fig. 3).

HepG2 cells were incubated for 48 h at 37°C with 100 µM verapamil (Vera), in each case 100 µM D617, D702, D703, 30 µM norverapamil (Nor) or 100 µM gallopamil (Gallo). After lysis of the cells, the β-glucuronidase activity was determined by means of 4-methylumbelliferyl-β-D-glucuronide cleavage (significant difference to the control, * $P < 0.01$, ** $p < 0.001$, $n = 3$ independent experiments).

Example 4

Lowering of the beta-glucuronidase expression by verapamil in the human hepatoma cell line HepG2 (Fig. 4).

HepG2 cells were incubated 48 h at 37°C with 100 µM verapamil or DMSO (control). After lysis of the cells, 50 µg cellular protein were separated off by means of SDS page, transferred to nitrocellulose and subsequently incubated with the monoclonal antibody 2156/42. The band intensity was determined densitometrically (DE = densitometric units; * significant difference to the control, $p < 0.05$; $n = 3$ independent experiments).

Inhibition of the glucuronidases in the rat intestine by verapamil (comparison)

In a study with Sprague-Dawley rats, the absorption of orally administered morphine-6-glucuronide (M6G) to two groups (group 1: $n = 5$, without verapamil administration; group 2: $n = 4$ previous verapamil administration) was investigated. The study was carried out with rats since these cannot form M6G from morphine (Aasmundstad T.A., Biochem. Pharmacol. (1993) 46: 961-968) so that the M6G measured in the plasma originated from the absorption of the orally administered M6G.

Whereas the previous administration of verapamil had no influence on the height of the plasma concentration of M6G or its variation in time, the concentrations of morphine and M3G in the case of previous verapamil administration (group 2) were distinctly smaller than in the case of the group without verapamil (group 1) (Fig. 5).

The absent influence on the height of the plasma concentration of M6G or its variation in time makes it improbable that the reduction of the morphine and M3G absorption depends upon an inhibition of the intestinal mobility (Shah M.H., J. Pharm. Pharmacol. (1987) 39: 1037 - 1038; Krevsky B., Dig. Dis. Sci. (1992) 37: 919 - 924). It is known that M6G inhibits the intestinal motility with the same potency as morphine (Schmidt N., Eur. J. Pharmacol. (1994) 255: 245 - 237). An increase of this inhibition by verapamil (Shah M.H., J. Pharm. Pharmacol. (1987) 39, 1037-1038) acts with all probability on M6G and morphine to the same extent. On the other hand, only the plasma level of morphine or M3G but not of M6G were reduced, i.e. the cleavage of M6G available after oral administration to morphine is thus inhibited. Therefrom result lower morphine and, as a result, M3G plasma levels since the greater part of the absorbed morphine is metabolised by glucuronyl transferases to M3G. The carrying out of the experiments is described in Example 5.

Example 5

Plasma concentration time progression of morphine-6-glucuronide (M6G), morphine and morphine-3-glucuronide (M3G) after oral administration to Sprague-Dawley rats of M6G with or without previous oral administration of verapamil (Fig. 5A and B)

The investigation was carried out on 9 male Sprague-Dawley rats. The rats were divided into 2 groups: group 1 (5 animals, weight: 258.6 ± 31.2 g) received only 62.5 mg/kg morphine-6-glucuronide (M6G) administered orally. Group 2 (4 animals, weight 272 ± 8 g) received, 15 minutes before M6G administration (62.5 mg/kg orally), 70 mg/kg verapamil orally administered. The groups did not differ significantly from one another with regard to their weight (t-test: t = -0.923, p = 0.401; confidence interval for difference group 1 - group 2: -51.6 to 24.8 g)

M6G nd verapamil were dissolved in Ringer lactate and subsequently mixed with tylose mucilage. To each rat were administered orally 62.5 mg M6G per kg body weight in tylose mucilage. 15 min before administration of M6G, 4 rats received 70 mg verapamil per kg body weight orally administered in tylose mucilage.

For the determination of the plasma concentrations of M6G, morphine and M3G, in the case of each rat 6 blood samples were taken (each about 200 µl) at the following times: before the administration of M6G, as well as 1, 2, 4, 6 and 8 hours after M6G administration. The blood samples were transferred into heparinised EDTA synthetic resin test tubes and immediately centrifuged. Until analysis, the prepared blood samples were stored at -20°C. The concentration of M6G, morphine and morphine-3-glucuronide (M3G) were determined by means of HPLC (cf. Hartley R., Biomed. Chromatog. (1993) 7: 34 - 37). The detection limit lay for all three substances at 10 ng/ml, i.e. 35.05 nmol/l for morphine and 22.45 nmol/l for the morphine glucuronides. In the whole calibration range, the variation coefficient in the whole calibration range (10 - 500 ng/ml) lay below 11%.

Inhibition of microbial beta-glucuronidase by verapamil

From Example 5 is to be seen that a cleavage of glucuronides (M6G) takes place in the intestines of the rat. It is not to be seen whether beta-glucuronidases of the rat and/or microbial beta-glucuronidases (e.g. E. coli) are responsible for this cleavage.

In order to clarify this question, beta-glucuronidases from rat intestine homogenates and from E. coli were incubated with verapamil or D-glucaric acid-1,4-lactone in the presence of 4-methylumbelliferyl-β-D-glucuronide (MUG). The cleavage of the 4-methylumbelliferyl-β-D-glucuronide is a measure for the activity of the beta-glucuronidase. As is to be expected, D-glucaric acid-1,4-lactone inhibits not only the beta-glucuronidase activity of

the rat intestine homogenates but also the E-coli beta-glucuronidase (Fig. 6A and B). Surprisingly, the bacterial enzyme was clearly inhibited by verapamil (IC_{50} = 30 µM), whereas the rat beta-glucuronidase is not measurably influenced by verapamil (Fig. 6A and B).

The carrying out of the experiment is described in Example 6.

Example 6

Inhibition of 4-methylumbelliferyl-β-D-glucuronide (MUG) cleavage by verapamil and D-glucaric acid-1,4-lactone (Fig. 6A+B).

Deep frozen tissue powder of a rat mucosa (duodenum and jejunum) was suspended in 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM pefabloc® (firm Roth, Karlsruhe, Germany). The protein concentration was determined according to the method of Lowry (Lowry O.H., J. Biol. Chem. (1951) 193: 265 - 275). The incubation and analysis took place according to: (Sperker B., J. Pharmacol. Exp. Ther. (1997) 281: 914 - 920). 50 µl incubation mixture contained 2.25 µg rat protein homogenate or 110 pg (0.001 units) purified E. coli beta-glucuronidase (firm Sigma, Deisenhofen, Germany). The test buffer contained 0.2 mM MUG (firm Sigma, Deisenhofen, Germany).

The incubation mixtures were mixed at 37°C with verapamil or D-glucaric acid-1,4-lactone. After 10 minutes, the MUG buffer was added. After 1 hour at 37°C, the enzymatic reaction was stopped by addition of 150 µl 200 mM sodium carbonate solution. After centrifuging (5 min., 13,000 r.p.m.), the supernatants were analysed by means of HPLC (fluorescence: absorption 355 nm, emission 460 nm). The enzyme activity was correlated with the liberation of 4-methylumbelliferone (MU). The experiments were carried out at the corresponding optima of the beta-glucuronidases (pH 7.0 E. coli or pH 5.0 rat). The results of Fig. 6 show that verapamil is not able

to inhibit the glucuronidase of the rat but is a good inhibitor for the bacterial glucuronidase from E. coli.

On the other hand, the known inhibitor D-glucaric acid 1,4-lactone inhibits both enzymes equally well.